35 Modulating Protein–Protein Interactions to Develop New Therapeutic Approaches

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INTRODUCTION

The manipulation of protein–protein interactions, which are involved in nearly all biological functions, is a potentially powerful tool for developing new therapeutic strategies. Several approaches have already been applied to develop improved promoters for gene therapy or to alter the tropism of viral vectors. This chapter describes some representative examples in this area of research.

TRANSCRIPTIONAL TARGETING

In the context of cancer gene therapy, transcriptional targeting allows the expression of a transgene in a controlled manner (Nettelbeck et al. 2000). In an ideal system, basal expression of the gene to be regulated should be low or undetectable but induced to high absolute levels by specific transcription factors affecting only targeted cells. For example, tumor cells, but frequently not the cells in the tissue of tumor origin, are proliferating. On the basis of this observation, we developed a strategy combining cell-cycle-regulated (Müller 1995) and cell-type-specific gene expression (Sikora 1993) in the same promoter. The basic idea of the strategy established in our labora-

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tory is to drive transcription of a transgene by an artificial heterodimeric transcription factor, whose DNA-binding subunit is expressed from a tissue-specific promoter, whereas the *trans*-activating subunit is transcribed from a cell-cycle-regulated promoter (Fig. 1) (Müller et al. 1996). The construction of this heterodimeric transcription factor is based on the modular structure of the transcription factor, which allows the combination of DNA-binding and *trans*-activation domains derived from different proteins. The critical features of such a heterodimeric transcription factor are (1) a DNA-binding domain that binds with high affinity to a sequence that is not present in the mammalian genome and is not recognized by endogenous transcription factors; (2) an activation domain that, when recruited to a promoter, induces high levels of transcription of the target gene; and (3) a target gene promoter that has little or no basal activity but promotes transcription to high levels on binding of the heterodimeric transcription factor. We have called this approach a *d*ual specificity *c*himeric *t*ranscription *f*actor (DCTF) system. Apart from the chosen promoters, the DCTF system is critically dependent on specific protein dimerization interfaces that cannot bind or be bound by endogenous factors.

To avoid any interference of the DCTF system with endogenous proteins, we first sought to identify a setting that would allow the assembly of an efficient *trans*-activating complex using nonmammalian proteins. We first tested the bacterial LexA protein as a transcriptional DNA-binding domain (LexA-DBD amino acids 1–202; Brent and Ptashne 1984; Lech et al. 1988) and the activation domain of the herpesvirus VP16 protein (amino acids 411–455; Triezenberg et al. 1988) in combination with dimerization interface of the yeast transcription factors Gal4p (amino acids 820–900) and Gal80p (amino acids 1–435) (Chasman and Kornberg 1990); of the heterodimerizing yeast proteins Ino2p (amino acids 213–305) and Ino4p (amino acids 1–151) (Nikoloff et al. 1992), or of yAP-1 (amino acids 89–136) (Moye-Rowley et al. 1989), which can form homodimers. As a reporter gene, we used the firefly luciferase gene. However, none of these combinations gave rise to detectable expression of the transgene, presumably due to insufficient interactions of the yeast transcription factors in the context of mammalian cells.

In a separate approach, we made use of the dimerization interface of human lymphoid proteins CD4 and $p56^{Lck}$, which have been shown to associate also in nonlymphoid cells (Simpson et al. 1989). The CD4/LCK dimerization system has an advantage over other mammalian proteins in that the former molecules are normally associated with the plasma membrane and expressed only in a very restricted number of cell types (Shaw et al. 1989; Turner et al. 1990), making interference (i.e., competition) with the heterodimer formation in the nucleus unlikely. CD4 is a 55-kD T-lymphocyte membrane glycoprotein mediating the interaction of T cells with antigen-presenting cells (Biddison et al. 1982; Swain et al. 1983). It is composed of an amino-terminal extracellular domain of 372 amino acids, a transmembrane domain of 23 amino acids, and

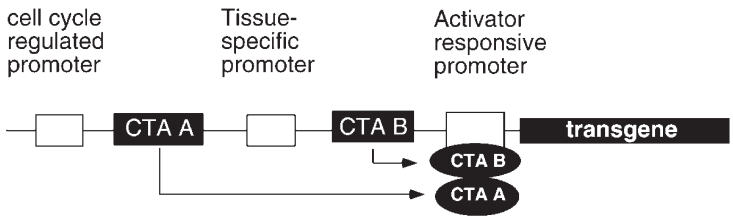

FIGURE 1. The dual-specificity chimeric transcription factor (DCTF) system. A DNA-binding subunit expressed from a tissue-specific promoter and a *trans*-activating subunit expressed from a cell-cycle-regulated promoter interact to form a heterodimeric chimeric transcriptional activator (CTA). The expression of a functional (i.e., heterodimeric) CTA will, therefore, be restricted to proliferating cells of a certain tissue type. The CTA binds and activates an activator-responsive promoter, thus leading to the expression of the transgene.

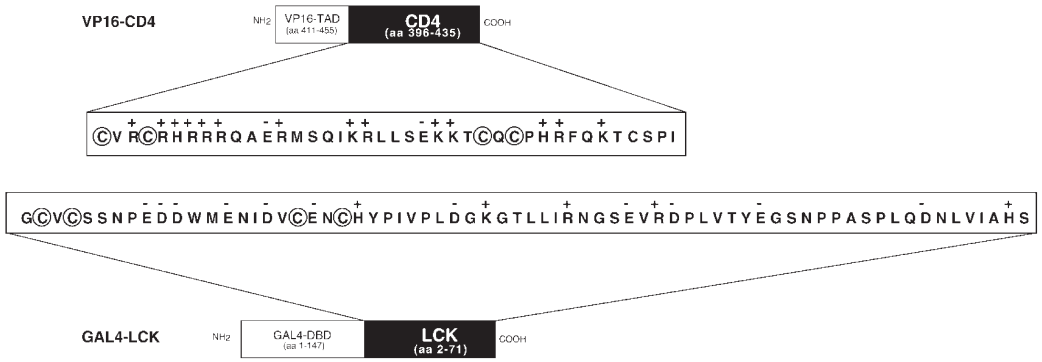

FIGURE 2. Structure of the CD4 and LCK chimeric transcription factors and a schematic representation of the GAL4–LCK and VP16–CD4 fusion proteins. The dimerization domains have been enlarged to show the sequence of the region. The acidic and basic residues that potentially make electrostatic interactions between subunits are indicated by a (–) or (+). The cysteine residues are circled. (+) Basic; (–) acidic.

a highly conserved cytoplasmic tail of 38 amino acids (Maddon et al. 1985; Littman et al. 1988). $p56^{Lck}$, a member of the src family of protein tyrosine kinases, is found predominantly in T lymphocytes (Marth et al. 1985; Voronova and Sefton 1986; Veillette et al. 1987). Structure–function analysis of mutant and chimeric molecules containing fragments of CD4 and $p56^{Lck}$ showed that the cytoplasmic tail of CD4 is necessary and sufficient for interaction with 32 amino-terminal residues of $p56^{Lck}$ (Shaw et al. 1989). Complex formation is critically dependent on the presence of free cysteines in the cytoplasmic tails of CD4 and in the amino-terminal domain of $p56^{Lck}$ but does not imply disulfide bonds (Rudd et al. 1988; Shaw et al. 1990). In addition, the charged amino acids surrounding the cysteine motif are predominantly basic in CD4 (+-+-X-Cys-X-Cys-Pro), where X is a nonconserved residue and + is a basic residue), and acidic in $p56^{Lck}$ (Cys-X-X-Cys), suggesting that salt bridges may stabilize an interaction centered on the cysteine residues (see Fig. 2).

Using CD4/LCK as the interaction interface, we demonstrated that the GAL4 DNA-binding domain (DBD; amino acids 1–147) (Webster et al. 1988; Chasman and Kornberg 1990) and VP16 *trans*-activation domain (amino acids 411–455; Lech et al. 1988) in combination with the interaction domains of CD4 (amino acids 396–435) and $p56^{Lck}$ (LCK; amino acids 2–71) (Shaw et al. 1989; Turner et al. 1990) were far superior to the yeast interaction domains mentioned above. The basic system was further optimized by the amino-terminal linkage of a nuclear localization signal (NLS) from SV40 T antigen (Kalderon et al. 1984) to both transcription factors. The addition of the NLS sequence to both transcription factors increased the overall activity of the system fivefold. Moreover, the insertion of ten instead of five GAL4-binding sites (Webster et al. 1988) into the luciferase vector also increased promoter activity fivefold.

To establish a regulatory mechanism that selectively operates in proliferating melanoma cells, the expression of the chimeric transcription factors was driven by the *cyclin A* promoter for S/G_2-phase-specific transcription (Zwicker et al. 1995) and a tyrosinase promoter/enhancer construct for melanocyte-specific expression (Shibata et al. 1992). The constructs used for the subsequent studies are shown in Figure 3. The tissue-specific transcription of the DCTF system was demonstrated by cotransfection experiments in proliferating tumor cell lines originating from three different carcinoma types. The tyrosinase promoter driving LCK gives rise to 10- to 20-fold higher levels of luciferase activity in melanoma cell lines compared to prostate and hepatoma cell lines. In addition, deletion of the CD4 interaction domain results in levels of expression representing the background activity of the system in all cases. Furthermore, we tested the DCTF system for simultaneous tissue-specific and cell-cycle-regulated expression. Tumor cells were synchronized in G_1 by methionine deprivation and cotransfected with the constructs described above.

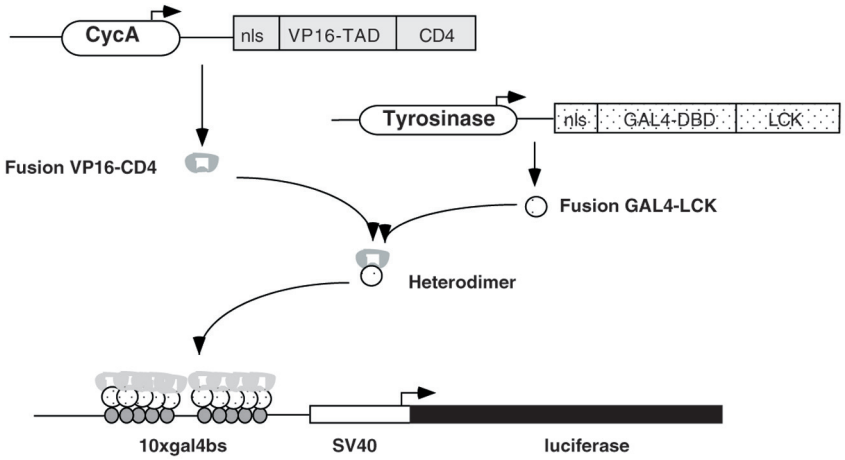

FIGURE 3. Outline of the experimental strategy. One subunit of an artificial transcription factor (CD4), consisting of the transcriptional activation domain of the herpes simplex virus protein VP16 (amino acids 411–455) and the cytoplasmic domain of the human CD4 (amino acids 396–435), is expressed from the cell-cycle-regulated cyclin A promoter. The second subunit (LCK), consisting of the GAL4 DNA-binding domain (amino acids 1–147) and the amino-terminal domain of the human $p56^{Lck}$ (LCK; amino acids 2–71), which interacts with the CD4 cytoplasmic domain, is expressed from the tissue-specific tyrosinase promoter. Only in proliferating cells originating from this tissue will both subunits be expressed and thus be able to form a complex through the CD4–LCK interaction. Binding of this heterodimeric transcription factor to an effector/reporter gene construct containing GAL4-binding sites (gal4bs) and the minimal SV40 promoter (nucleotides –56 to +40) will then lead to transcriptional activation through the strong VP16 activation domain. (CycA) Cyclin A; (nls) nuclear localization signal; (TAD) *trans*-activation domain.

Determination of luciferase activities in growing and G_1-arrested cells clearly demonstrated a preferential expression in the proliferating melanoma cells. Cell-type specificity was in the range of 10-fold and cell cycle regulation approximately 5-fold (Jérôme and Müller 1998). The extent of regulation was further increased (1.5-fold) by cloning the two expressing cassettes into the same plasmid, a prerequisite for future testing of the DCTF system in animals. Although these results demonstrated the suitability of the DCTF system for directing gene expression to proliferating tumor cells, the extent of the regulation had to be improved further for a potential application in cancer gene therapy. Because the CD4–LCK interaction is weak, and potential interactions with endogenous molecules might result in the sequestration of the chimeric transcription factor used in the DCTF system, we reasoned that the replacement of the heterodimerization interface domains might enhance the overall performance of the system.

Leucine zippers (LZiPs) represent strong interaction domains commonly found in transcription factors (Busch and Sassone-Corsi 1990). They have been shown to be functional in a heterologous context and form particularly strong interactions (Kouzarides et al. 1989; Neuberg et al. 1989; Schmidt-Dörr et al. 1991). Dimerization through LZiPs is mediated by regularly spaced leucines (heptad repeats) in parallel α-helices through hydrophobic interactions, whereas the choice of the dimerization partner is determined by other amino acids, mainly charged residues forming salt bridges (Kouzarides et al. 1988; O'Shea et al. 1989, 1992; Schuermann et al. 1989, 1991; Cohen and Parry 1990; Oas et al. 1990). For the Fos and Jun transcription factors, the preferential formation of the heterodimer over either of the homodimers is brought about by the positively charged amino acids in the Jun LZiP and the negatively charged residues in the Fos counterpart, leading to strong self-repulsion of both proteins (O'Shea et al. 1992; Glover and Harrison 1995). The strength and specificity of the interaction between Fos and Jun, as well as their preferential heterodimerization, suggested that they could be useful for the improvement of the DCTF system. Both Jun and Fos family members are expressed to variable extents in all cells. To make

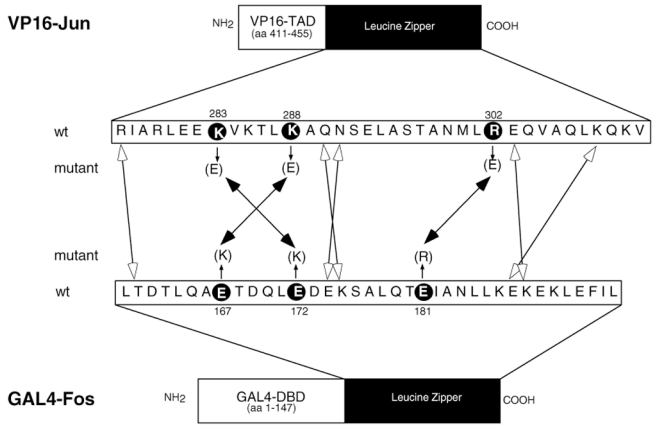

FIGURE 4. Structure of the chimeric transcription factors. Schematic representation of the GAL4–fos and VP16–jun fusion proteins. The leucine zipper structures have been enlarged to show the sequence of the region. Arrows connect the residues making electrostatic interactions between subunits. Black arrows link the amino acids switched from one zipper to the other, and the mutants generated are indicated in brackets. (Reprinted, with permission, from Jérôme and Müller 2001.)

the Jun/Fos LZiPs suitable for the DCTF system, it was therefore necessary to prevent interactions between the chimeric transcription factors and the endogenous Jun and Fos proteins. Using the resolved crystal structure (Glover and Harrison 1995), we introduced three acidic amino acids from c-Fos into the c-Jun LZiP (mJun) and three corresponding basic amino acids from c-Jun into the c-Fos LZiP (mFos), as depicted in Figure 4, thereby creating mixed-charged structures. Analysis of the oligomerization properties in an in vitro translation/association assay showed that these mutated zippers interact specifically with each other, but not with the wild-type LZiPs. After insertion into the transcription factors used in the DCTF system in place of the CD4 and $p56^{Lck}$ sequences, these new heterodimerization domains enhanced promoter activity >100-fold to a level that makes this system suitable for applications in gene therapy. Moreover, the analysis of the fos/jun DCTF system in an in vivo setting of experimental melanoma showed a specific expression that was almost 4-fold higher than the expression of a SV40 construct after intratumoral injection of naked DNA (Jérôme and Müller 2001).

The DCTF system opens up the possibility of designing a particularly efficient system for *g*ene-*d*irected *e*nzyme *p*rodrug *t*herapy (GDEPT). A promoter could be restricted to the proliferating cells of a defined tissue type (i.e., tumor cells) to direct the expression of an enzyme that cleaves a prodrug to a toxic drug that is not cell-cycle-dependent and gives rise to a strong bystander effect. This should lead to the destruction, not only of the expressing tumor cell, but also of all adjacent cells irrespective of their proliferative activity. In contrast, the commonly used thymidine kinase/ganciclovir system leads only to a selective destruction of the proliferating cells in the tumor (Conners 1995; Rigg and Sikora 1997), which is a major drawback in view of the fact that a substantial fraction of tumor cells is in a resting state or a prolonged G_1 phase at any given time. Moreover, instead of a prodrug-activating system it might also be possible to use transgenes encoding secreted cytotoxic proteins or cytokines stimulating a tumor-directed immune response. In addition, the mJun/mFos LZiP domains should also be useful for addressing other problems, as, for example, directing partner proteins to a specific subcellular location (Dang et al. 1991), to increase the activity of multienzyme systems by virtue of a physical link between the enzymes (Davidson et al. 1993; Gontero et al. 1993) or to improve the function of heterologous proteins in a gene therapeutic setting by enabling oligomerization (Walczak et al. 1999).

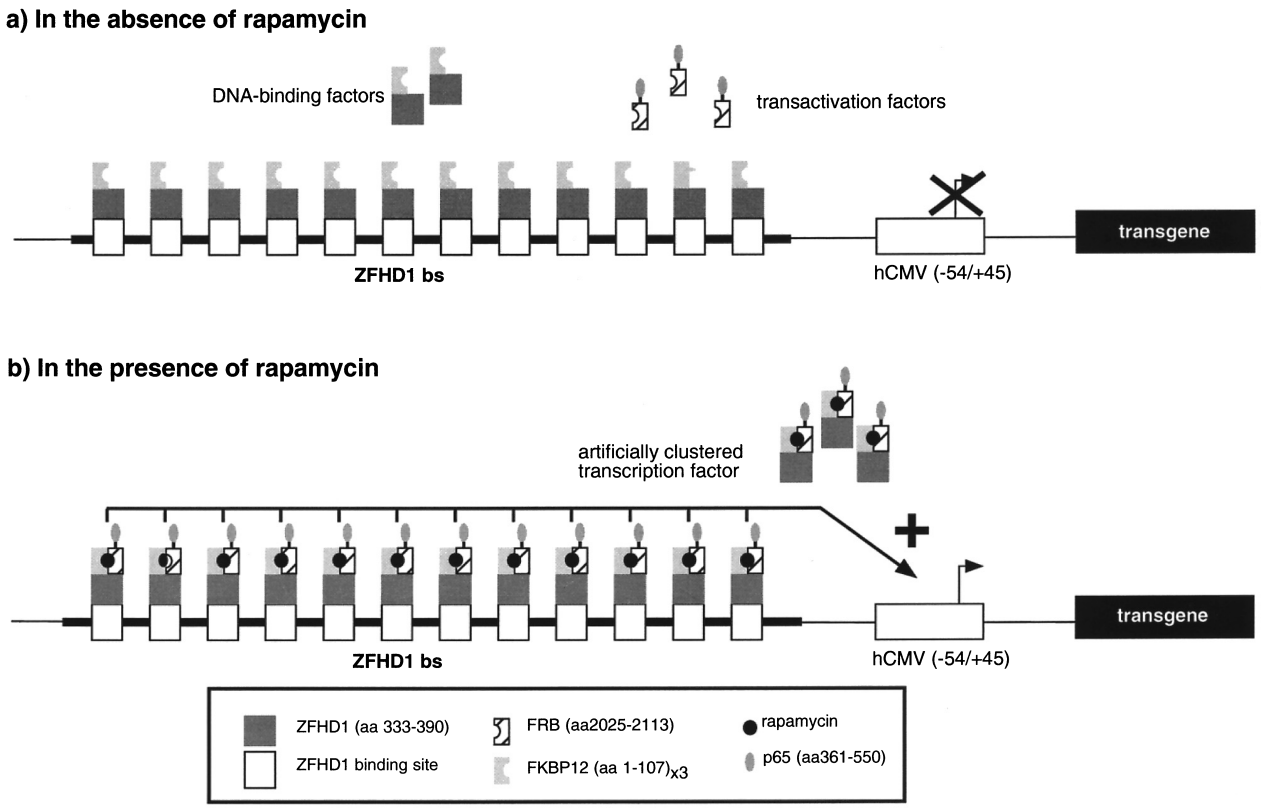

FIGURE 5. The *r*apamycin-*i*nducible *h*umanized *s*ystem (RIHS). (*a*) In the absence of rapamycin, the two components of the *trans*-activator do not interact with each other and transcription does not proceed. (*b*) In the presence of rapamycin, the FKBP12-rapamycin binding (FRB) domain interacts with FKBP12. Rapamycin-mediated association of the domains results in a fully functional transcription factor that binds to and activates expression of a target gene containing binding sites for ZFHD1.

INDUCIBLE TRANSCRIPTION

In a clinical setting, a system that can be switched on and off at will to allow the timing of delivery and the optimization of gene product dosage would be highly desirable. This is particularly important for studies involving genes encoding toxic or otherwise highly bioactive proteins. Several systems regulatable by small molecules have been described, including promoter systems that are inducible by tetracycline (Baron and Bujard 2000), ecdysone (No et al. 1996), RU486/mifepristone (Wang et al. 1997), or FK506/rapamycin (Rivera et al. 1996). Here, we focus on the latter, because this is the most advanced system with respect to gene therapy.

The goal of a "gene switch" is to place the expression of a gene of interest under control of a transcription factor, the activity of which can be regulated with a small-molecule drug (Rivera et al. 1996). The cyclophilin and immunophilin (FKBP12-rapamycin-associated protein; FRAP) protein families and the FK506-binding proteins (FKBPs) contain chemical-binding domains that can be fused to DNA-binding and *trans*-activation domains. The dimerization of these chimeric transcription factors can be achieved via chemical inducers of dimerization (CIDs), which are the immunosuppressors FK506, rapamycin, and cyclosporin A (CsA) (Fig. 5) (Spencer et al. 1993; Klemm et al. 1998). These cell-permeable molecules display pharmacological properties that favor their use in vivo. They are used to control the interaction of a transcription activation domain and a DNA-binding domain and to allow the level of expression of a target gene to be regulated in a dose-responsive manner by the concentration of dimerizer independently of normal cellular physiology.

One of the most promising systems is a humanized version (Rivera et al. 1996) of the previously published system (Belshaw et al. 1996; No et al. 1996). A DNA-binding function is provided by a chimeric DNA-binding domain (ZFHD1; amino acids 333–390 of Zif268 fused to amino acids 378–439 of Oct-12 and spaced by 2 Gly residues) that is not recognized by endogenous transcription factors in the experimental context (Pomerantz et al. 1995). Three tandemly repeated copies of human FKBP12 (amino acids 1–107; Bierer et al. 1990) were fused to the ZFHD1 DNA-binding domain. A subdomain of p65 NF-κB (amino acids 361–550; Schmitz and Baeuerle 1991) is used as a transcriptional activator moiety and the small molecule binding function by a domain derived from the human FRAP (amino acids 2025–2113) (Brown et al. 1994). These two chimeric transcription factors have no inherent affinity to one another in the absence of rapamycin.

Using this system, a precise control of circulating protein levels in vivo from implanted, stably transfected cells secreting human growth hormone (hGH) in response to stimulation by rapamycin could be achieved. The combination of genetically engineered cells and the oral administration of a therapeutic compound to which the cells are responsive should permit the safe and prolonged delivery of therapeutic proteins (Rivera et al. 1996). Recently, the exploitation of the *r*apamycin-*i*nducible *h*umanized *s*ystem (RIHS) to express the murine erythropoietin in mice and nonhuman primates after adeno-associated virus (AAV) transfer demonstrated the ability to titrate in vivo expression of the transgene with the dose of rapamycin (Ye et al. 1999).

To summarize, the RIHS displays the following advantages: (1) It is, in principle, applicable to human gene therapy because it is composed completely of human proteins; (2) there is very low background activity in vitro and in vivo, and a high induction ratio independent of host physiology and cell-type-specific factors; (3) it has a small DNA-coding capacity required for the regulatory proteins (<2.5 kb); and (4) the system's modularity allows each component to be optimized and engineered independently. Furthermore, in contrast to bacterial repressors (Gossen et al. 1992), which rely on allosteric intramolecular interactions to control DNA-binding activity, the RIHS can be applied to virtually any DNA-binding and activation domain. Nonetheless, some drawbacks might be the slow kinetics of de-induction and the growth-inhibitory and immunosuppressive effects of rapamycin. A potential solution to the latter problem may be the use of new nonimmunosuppressive analogs of rapamycin (Liberles et al. 1997; Harvey and Caskey 1998). Thus, the RIHS may become a method of choice for applications in human subjects.

RETARGETING OF VIRAL VECTORS

Viral vectors derived from retroviruses, adenoviruses, adeno-associated viruses, and herpesvirus are frequently used as gene delivery vehicles because of their capacity to carry foreign genes and their ability to deliver and express these genes efficiently (for review, see Walther and Stein 2000). Recently, efforts have been made to improve viral targeting based on control of protein–protein interactions, which are likely to play an essential role in promoting efficient and tolerable gene therapy protocols. The following section gives a short overview of the progress made in recent years within this field.

Retroviruses often suffer from a low efficiency of infection (Wang et al. 1991). One way to remedy this problem could be to alter the cell tropism of these viruses by incorporating receptor ligands or single-chain antibodies into the envelope protein (Young et al. 1990; Russell et al. 1993; Kasahara et al. 1994; Valsesia-Wittman et al. 1994; Cosset et al. 1995; Han et al. 1995; Somia et al. 1995; Marin et al. 1996; Schnierle et al. 1996; Ager et al. 1996; Chu et al. 1997). Although retargeting could be demonstrated, viral titers were generally very low, which represents a serious obstacle with respect to their applicability. One explanation might be that only specific cell-surface molecules on target cells are able to mediate efficient infection. Another approach consists of the introduction of protease target sequences or spacer peptides between the Env protein and the ligand introduced for retargeting (Valsesia-Wittman et al. 1997; Peng et al. 1997, 1998, 1999). Although this retargeting approach was more successful, numerous technical problems still

remain to be solved before retargeted retroviral vectors can be used in a clinical setting of gene therapy.

Replication-deficient adenoviruses have several key attributes that make them potentially useful for clinical gene therapy. These include their large packaging capacity, the relative ease of virus production at high titers, and their ability to infect both dividing and nondividing cells. There are, however, also important limitations, including its widespread tropism, short-term transgene expression, and stimulation of inflammatory and immune responses.

Adenovirus infection is initiated by high-affinity binding of the fiber protein "knob" domain to the coxsackie-adenovirus receptor (CAR) (Bergelson et al. 1997; Tomko et al. 1997). The virion subsequently binds via its penton capsid protein to a cellular integrin receptor (via a RGD motif) and internalizes via the receptor-mediated endocytosis pathway (Bilbao et al. 1998). A low level of CAR expression on target cells has emerged as a key limiting factor in adenovirus gene delivery to certain neoplastic (Dmitriev et al. 1998; Miller et al. 1998; Blackwell et al. 1999; Kasono et al. 1999) and nonneoplastic (Zabner et al. 1997; Kaner et al. 1999; Walters et al. 1999) cells. Additionally, expression of CAR in non-target tissues may lead to undesired transgene expression with risk of toxicity (Yee et al. 1996). Therefore, it is important to target the delivery and/or expression of adenovirus-encoded transgenes to the appropriate set of cells. Moreover, sequestration of adenovirus vectors in the liver is a key limiting factor to the use of these agents via the systemic route (Van der Eb et al. 1998).

On the basis of the well-understood mechanism of adenoviral cell entry, several groups are working on approaches toward an "immunologic" or a "genetic" retargeting of adenoviral vectors (Douglas and Curiel 1995; Curiel 1999). Immunologic retargeting strategies are based on the use of bispecific conjugates (antibody directed against a component of the virus and a targeting antibody or ligand) and require both the abolition of native targeting and the introduction of a new tropism (Fig. 6). For example, a folate-conjugated neutralizing antibody against the knob region of adenovirus enabled the targeting of several tumor types (Weitman et al. 1992). Nettelbeck et al. (2001) developed a bispecific single-chain diabody directed against endoglin and the adenovirus knob domain, which restricts and enhances the adenoviral transduction to endothelial cells. Another approach made use of a fusion protein between a neutralizing anti-knob scFv and the epidermal growth factor (EGF). This "adenobody" was successfully used to retarget an adenoviral vector to EGF receptor-positive tumor cells (Watkins et al. 1997). Interestingly, this pathway of infection appears to bypass the need for penton base/integrin interaction for internalization of the virus. More recently, Reynolds and coworkers published the first in vivo data demonstrating that an immunological retargeting approach can modify the tropism of a systemically injected adenovirus (Reynolds et al. 2000).

Genetic retargeting involves the modification of the capsid proteins at the level of the viral genome (for review, see Bilbao 1998; Curiel 1999). Genetic modifications can be directed to the

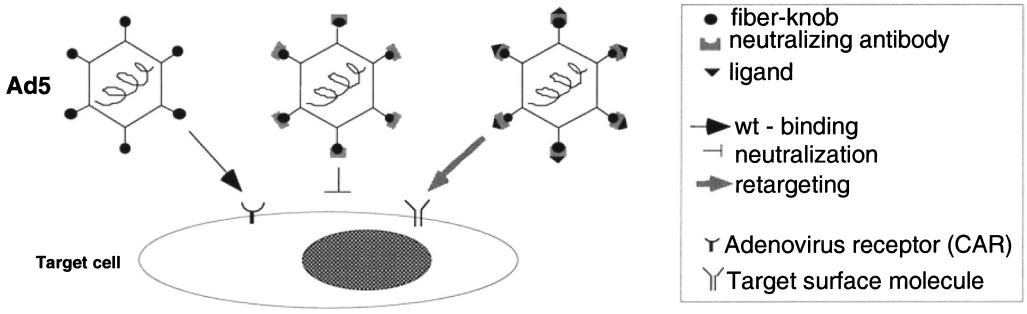

FIGURE 6. Immunologic retargeting strategy. An antibody directed against the knob domain can neutralize the adenoviral binding of the fiber-knob to the CAR receptor. The fusion of a targeting protein or ligand to this anti-knob neutralizing antibody allows a selective binding of an adenovirus to the target tissue.

fiber, to the penton base, or to capsid proteins (Wickham et al. 1995). Wickham et al. (1996) showed that incorporating a high-affinity peptide ligand for α_V integrin into the adenoviral fiber protein can enhance adenoviral transfection efficiency in endothelial cells in vitro and in vivo (Wickham et al. 1997a,b; McDonald et al. 1999). Retargeting can also improve the transducibility of certain tissues, as shown for Kaposi's sarcoma (Goldman et al. 1997). Importantly, retargeting should allow a substantial reduction of the viral dose required to achieve a therapeutic effect, thus potentially reducing toxic side effects, which are a major concern with respect to adenoviral vectors (Schulick et al. 1995a,b).

SUMMARY

Manipulations of protein–protein interactions to develop new therapeutic strategies have been extensively developed within the past few years. The work of several groups provides new tools, based on transcriptional targeting, that allow restricted expression of a transgene to a defined tissue type (DCTF system; Jérôme and Müller 1998, 2001), or a controlled expression of a transgene via cell-permeable molecules (RIHS; Rivera et al. 1996). Other groups successfully modified the adenoviral tropism via immunologic or genetic retargeting (for review, see Curiel 2000). In the near future, one can predict that researchers will focus their work on improving these existing tools further, and combining transcriptional targeting with adenoviral retargeting; thus achieving a tightly controlled and localized transgene expression in vivo, making these systems suitable for human gene therapy. Above all, the main goal of cancer gene therapy is to provide tools to cure cancer avoiding the side effects of the currently used protocols.

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